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Applicant: Tagawa et al.

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DECLARATION UNDER 37 C.F.R. § 1.132 OF TOSHIAKI TAGAWA, Ph.D.

I, Toshiaki Tagawa, Ph.D., do hereby declare:

1. I am a co-inventor of the subject matter disclosed and claimed in the above-captioned patent application.

2. I graduated from Kyusyu University, graduate school of science, with a Master's degree in 1984. I earned a Ph.D. from Toho University School of Medicine in 2003.

3. I am an employee of Mitsubishi Tanabe Pharma Corporation, which is the owner of the above-captioned patent application, and I joined the company and have been engaged in the research of drug delivery systems since 1989. I am a member of the Japan Society of Drug Delivery System.

4. I am a co-author of several published scientific articles including:

"Establishment and evaluation of cancer-specific human monoclonal antibody GAH for targeting chemotherapy using immunoliposomes," *Hybridoma and Hybrimomics, 23*: 109-120 (2004).

"Antitumor effect of MCC-465, pegylated liposomal doxorubicin tagged with newly developed monoclonal antibody GAH, in colorectal cancer xenografts," *Cancer Science, 95:* 608-613 (2004).

"Efficacy of immunoliposomes on cancer models in a cell-surface-antigen-density-dependent manner," *British Journal of Cancer, 89:* 1545-1551 (2003).

"Characterisation of LMD virus-like nanoparticles self-assembled from cationic liposomes, adenovirus core peptide μ(mu) and plasmid DNA," *Gene Therapy, 9:* 564-576 (2002).

"Immunoliposomes bearing polyethylenglycol-coupled Fab' fragment show prolonged circulation time and high extravasation into targeted solid tumors in vivo," *FEBS Letters, 413:* 177-180 (1997).

"Targeting efficiency of PEG-immunoliposome-conjugated antibodies at PEG terminals," *Advanced Drug Delivery Reviews, 24:* 235-242 (1997).

"Improvement of therapeutic effect by using Fab' fragment in the treatment of carcinoembryonic antigen-positive human solid tumors with adriamycin-entrapped immunoliposomes," *Japanese Journal of Cancer Research, 85:* 434-440 (1994).

5. I conducted an experiment in evaluate the encapsulation efficiency of a water soluble marker, 5(6) carboxyfluorescein, in the mixed liposome prepared following the method described in Example 7 of U.S. Patent 6,193,997 (Modi). I used 5(6) carboxyfluorescein as a typical water soluble marker, which is shown to be encapsulated in liposomes and employed for a variety of studies on liposome characterization.

Materials

6. The following materials were used in the experiment: 5(6) carboxyfluorescein (Wako Chemical); sodium hyaluronate (Wako Chemical); sodium lauryl sulphate (Wako Chemical); glycolic acid (Wako Chemical); propylene glycol (Wako Chemical); and saturated lecithin (NC-21; NOF Corporation)

Experimental Methods

7. Mixed liposome was prepared according to the method of Example 7 of Modi, except that 5(6) carboxyfluorescein (CF) was used instead of insulin and the experimental scale was reduced to 1/10.

8. Specifically, 10 mg of sodium lauryl sulfate was added to 1 ml of CF solution (5 mM) and dissolved to obtain Solution A. Sodium hyaluronate (10 mg), 50 mg of glycolic acid, and 0.05 mL of propylene glycol were added to purified water (5 ml) and dissolved, and then the resulting solution was mixed with Solution A. Acidic precipitations of CF were formed with glycolic acid. The precipitation of CF were dissolved with 1 M NaOH solution for neutralization (total volume: 6.61 mL). The obtained mixture was filled in a glass pressure syringe (Avanti Pola Lipid), and the mixture was injected in 0.17 mL of 1% saturated lecithin solution at a rate of 1 mL within 1 second to obtain mixed liposome. In this connection, Example 7 of Modi is silent about a volume of 1% saturated lecithin. However, Modi discloses at column 6, lines 26-28, that "[t]ypically the ratio of the membrane mimetic amphiphile aqueous solution to the phospholipid solution is about 5:1 to about 20:1." Consistent with the above teaching by Modi, I used 0.17 mL of 1% saturated lecithin for 1 mL of the above obtained mixture.

9. The mixed liposome obtained was diluted 10-fold with phosphate buffered saline and subjected to a centrifuge ultrafiltration (Amicon Ultra apparatus, molecular weight cut off: 10 kD, Millipore), and then CF concentrations and lecithin concentrations before and after the ultrafiltration were determined. For quantification of CF concentration, absorption at 492 nm was measured. Concentrations of the lipid were calculated from peak areas obtained by liquid chromatography (HPLC) with charged aerosol detection.

Results and Discussion

10. CF concentration in the mixed liposome solution before the ultrafiltration was 63 μM, whilst CF concentration in the solution after ultrafiltration, i.e., after the passage of the ultrafiltration membrane, was 62 μM (n=3), indicating that most of the CF in the solution was found in a fraction of molecular weight of 10 kD or lower. Further, in order to verify that the mixed liposome did not pass through the ultrafiltration membrane, the concentration of lecithin in the filtrate was measured. Lecithin in the mixed liposome (0.201 mg/mL) was not detected in the filtrate obtained by the ultrafiltration (n=3, quantification limit: 0.03125 mg/mL).

11. The experimental results demonstrate that, in the mixed liposome encapsulating CF according to the method of Modi, 98% or more of the CF is present outside the mixed liposome, while only about 1% of the CF is encapsulated in the vesicle.

12. I hereby declare that all statements made herein of my own knowledge are true, that all statements made on information and belief are believed to be true, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: October 13, 2009 Toshiaki Tagawa

Toshiaki Tagawa, Ph.D.